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Patentanmeldung Nr. Patent application No. Demande de brevet n°

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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office
Le Président de l'Office européen des brevets
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Pediocin-producing pediococci

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PEDIOCIN-PRODUCING PEDIOCOCCI

TECHNICAL FIELD OF THE INVENTION

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The present invention relates to pediocin-producing pediococci isolated from human faeces, in particular *Pediococcus acidilactici* isolated from faeces for use in the gastrointestinal tract of humans to provide a health promoting action, in particular against infections by multi-resistant pathogens.

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BACKGROUND OF THE INVENTION

Intestinal diseases such as intestinal infections are usually caused by bacteria or viruses. Such bacterial infections can be treated with synthesised antibiotics.

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Synthesised antibiotics are chemical compounds that will kill pathogenic bacteria. However, a problem encountered with such antibiotics is that after regular administration to the patient, the organism begins to build up a certain resistance to said antibiotics, thus requiring either the use of a stronger synthesised antibiotic or the use of an alternative solution to treat the infection.

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Alternative solutions can be represented by the use of probiotics. Probiotics are viable bacteria that beneficially affect the host by improving its intestinal microbial balance. These bacteria can have a prophylactic and/or a therapeutic effect on intestinal diseases, such as intestinal infections. Thus, when these micro-organisms are administered to humans or animals they can compete with pathogenic bacteria for nutrients and/or inhibit adhesion sites on the intestinal wall, as a result of which the number of pathogenic bacteria will decrease and infections are prevented or reduced. Further, probiotics produce organic acids, thereby lowering the pH, and thereby resulting in an antipathogenic action.

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Some bacteria can produce anti-microbial proteins or peptides, called bacteriocins. These anti-microbial components can inhibit the growth of bacteria by impairing the

cytoplasmic membrane of sensitive bacteria resulting in disturbances of the intracellular homeostasis.

5 Yet, not all bacteriocins have the same spectrum of activity. Hence, nisin which is a cationic peptide antibiotic produced by *Lactococcus lactis* has a very broad spectrum of activity, that is one which is detrimental to both beneficial strains such as lactobacilli and bifidobacteria as well as pathogenic strains in the intestine.

10 Accordingly, there is a need for a material that is more selective to the pathogenic species than to the beneficial species present in the intestine. By pathogenic strains, it is meant Gram-positive bacteria, in particular those of the enterococci type like *Enterococcus faecalis*, *Enterococcus faecium*, but also *Listeria monocytogenes* and mixtures thereof as well as Gram-negative bacteria, in particular those belonging to the genera *Klebsiella*, *Pseudomonas*, *Shigella* and mixtures thereof.

15

Further, with the emergence and wide geographic spread of multidrug resistant Gram-positive pathogens, the necessity for alternative antibacterial agents has become urgent. Typical of such multidrug resistant Gram-positive pathogens are the multi-resistant pathogens, in particular the vancomycin-resistant enterococci, hereinafter called VRE. The problem of infection caused by such VRE is even more acute in hospitals, wherein patients weakened by surgery or diseases are more sensitive to such pathogens.

25

Accordingly, there is also a need for a material that is effective against VRE.

Recently, the action of nisin has been studied in "Journal of Antimicrobial Chemotherapy" (1998) 41, 341-347 and was found to have a wide and powerful bactericidal effect, in particular against vancomycin-resistant isolates of *E. faecium* and *E. faecalis*.

30

However, for the reasons stated above, nisin is not a desired material for use herein due to its non-selective activity spectrum, and thus being to some extent detrimental to the intestinal florate equilibrium.

Besides VRE, the presence of non-vancomycin resistant enterococci in the GI tract can also be a cause of intestinal diseases. Accordingly, there is a need for a material that is also effective against non-vancomycin-resistant *Enterococcus*, especially *E. faecalis* as well as *E. faecium*.

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- Accordingly, there is also a need for a material which is effective against the pathogenic species present in the intestine, in particular against the pathogenic Gram-positive species, more in particular against enterococci, including VRE, *Enterococcus faecalis*, *Enterococcus faecium*, against *Listeria monocytogenes* and mixtures thereof as well as against the pathogenic Gram-negative species, in particular those belonging to the genera *Klebsiella*, *Pseudomonas*, *Shigella* and mixtures thereof, whilst not being detrimental to the intestinal flora.

- Still another problem arising with the presence of pathogens is the overgrowth of said pathogen in the gut, which causes diarrhea as well as secondary associated disorders. Typical of such secondary disorder associated with overgrowth of pathogens are selected from water disturbances, mineral balance disturbances, malnutrition, dysfunctioning of tissues, dysfunctioning of organs, dysfunctioning of organism, and mixtures thereof. The disturbances and malnutrition can in turn cause one or more of the following: nausea, vomiting, sickness but also endotoxemia, as well as sepsis ("blood-poisoning"), whereas the dysfunctioning mentioned above can in turn cause one or more of the following: death, infections, ulcers, bad wound healing, both in the gut as well as topically.

- Accordingly, there is also a need for a material that is effective against overgrowth of pathogens and thereby the associated secondary disorders.

- It has now surprisingly been found that strains from *Pediococcus* that have a specific survival rate in the gastrointestinal tract, preferably strains from pediocin-producing *Pediococcus*, in particular strains of *Pediococcus acidilactici*, more in particular the *Pediococcus acidilactici* strains isolated from human faeces, fulfil the above-mentioned needs. *Pediococcus acidilactici* isolated from human faeces has been deposited at the

Belgian Co-ordinated Collections of Microorganisms, Laboratorium voor Microbiology Gent (BCCMTM/LMG) on 24 April 2003 under No. LMG P-21927.

Pediocin produced by *Pediococcus* strains of the invention has been found to be
5 particularly effective for inhibiting in the gastrointestinal tract the growth of pathogenic Gram-positive strains of the enterococci type, preferably selected from vancomycin-resistant enterococci, *Enterococcus faecalis*, *Enterococcus faecium*, and mixtures thereof, while *Pediococcus* strains of the invention, in particular pediocin-producing
10 *Pediococcus* strains, have been found to be particularly effective for inhibiting in the gastrointestinal tract the adhesion of pathogenic Gram-negative strains, in particular those selected from the genera *Klebsiella*, *Pseudomonas*, *Shigella* and mixtures thereof.

In addition, the pediocin produced from the strain *Pediococcus acidilactici* LMG P-21927 according to the present invention has been found to be particularly effective for
15 inhibiting in the gastrointestinal tract the growth of pathogenic Gram-positive strains, in particular those selected from enterococci types, preferably selected from vancomycin-resistant enterococci, *Enterococcus faecium*, and *Listeria monocytogenes* and mixtures thereof, while the strain *Pediococcus acidilactici* LMG P-21927 according to the present invention has been found to be particularly effective for
20 inhibiting in the gastrointestinal tract the adhesion of pathogens Gram-negative strains, in particular those selected from the genera *Klebsiella*, *Pseudomonas*, *Shigella* and mixtures thereof.

Pediocin-producing *Pediococcus* strains are known in the art. They are described in
25 FEMS Microbiology Reviews 24 (2000) 85-106 by S. Ennahar et al. as well as in Natural Food Antimicrobial Systems 19 (2000) 525-566 by B. Ray and K. W. Miller. However, both publications describe pediocin-producing pediococci as being isolated from meat or plant. In that respect, it has been found that such isolates do not optimally perform when used in the human GI tract. Hence, the overall survival in the
30 gastrointestinal tract has been found lower than with isolates of the invention.

SUMMARY OF THE INVENTION

It is an aspect of the present invention to provide the use of pediococci for the manufacture of a composition for inhibiting pathogens strains in the gastrointestinal tract, wherein the pediococci are characterised by a survival rate as per the Survival rate Test defined herein of at least 80 %, preferably of at least 90 %, most preferably of at least 96 %.

It is another aspect of the present invention to provide the use of such pediococci and/or bacteriocin produced from pediococci for the manufacture of a composition for inhibiting in the gastrointestinal tract the growth of Gram-positive strains of the enterococci type.

It is also an aspect of the present invention to provide the use of pediococci and/or bacteriocin produced from pediococci isolated from human faeces for the manufacture of a composition for inhibiting in the gastrointestinal tract the growth of Gram-positive strains.

It is a further object of the present invention to provide the use of such pediococci for the manufacture of a composition for inhibiting in the gastrointestinal tract the adhesion of Gram-negative strains.

In another aspect of the present invention, there is provided a probiotic compound, wherein said probiotic compound is said pediococci.

In a further aspect of the present invention, there is provided a health-promoting action composition comprising said probiotic compound, and preferably with a compound selected from pediocin as herein defined, additional probiotics, prebiotics, immunoglobulines, and mixtures thereof.

DETAILED DESCRIPTION OF THE INVENTIONApplication

5 1)-Pediococci and bacteriocin produced from pediococci

Pediococci that are characterised by a survival rate as per the Survival rate Test defined herein of at least 80 %, preferably of at least 90 %, most preferably of at least 96 % are an important characteristic of the present invention.

10 Indeed, it has surprisingly been found that the pediococci according to the present invention present an overall survival in the GI-tract, in particular in the small intestine after the passage in the stomach, that is superior to pediococci isolated from plant or meat. This is demonstrated hereinafter in Table 3 of the examples.

15 **Survival rate Test**

The Survival rate Test is designed to evaluate strains which will survive both in the stomach and small intestine. For the purpose of the invention, the strains encompassed by the present invention are those which after contact for 3 h at 37 °C under anaerobic conditions in a medium that represents the stomach, and thereafter exposed for 3 h at 20 37 °C under anaerobic conditions to a medium that represents the small intestine have a survival rate of at least 80%, preferably of at least 90%, and more preferably of at least 96% in the small intestine.

The Survival rate Test is as follows:

25 The bacteria to be tested is grown in MRS for 24 hours and subsequently re-inoculated for 18 hours in MRS at 37 °C. 1 ml of the grown culture is added to 9 ml of reconstituted stomach medium, consisting of 8.3 g/l bacteriological peptone, 3.1 g/l NaCl, 0.11 g/l CaCl₂, 1.1 g/l KCl, 0.6 g/l KH₂PO₄, 22.2 mg/l pepsin and 22.2 mg/l lipase, pH 3.0 (measured at 20°C). The bacteria are incubated for 3 hours at 37°C in the 30 reconstituted stomach medium. Afterwards 1 ml of the incubated stomach medium with the bacterium is mixed with 9 ml of reconstituted small intestine medium and incubated for another 3 hours at 37°C. The reconstituted small intestine medium consists of 5.7 g/l bacteriological peptone, 1.25 g/l NaCl, 0.055 g/l CaCl₂, 0.15 g/l KCl, 0.68 g/l

KH_2PO_4 , 1.0 g/l NaHCO_3 , 0.3 g/l Na_2HPO_4 , 0.7 g/l glucose, 20.3 g/l pancreatin and 5.5 g/l bile, pH 6.5 (measured at 20 °C). Samples are taken at t=0, 3, and 6 hours and plated on MRS agar to determine the colony forming units.

- 5 The % survival in the small intestine is determined as 100 x (cfu's present in 10 ml small intestine medium/cfu's present in 1 ml stomach medium).

- 10 Preferred pediococci are the pediocin-producing pediococci that fulfil the survival test requirement above mentioned, more prefered are the pediocin-producing pediococci that are isolated from human faeces.

- 15 The pediococci strains, isolated from the human faeces, of the present invention that are able to produce pediocin are used as a probiotic. In the gastro-intestinal tract these strains will, after colonisation, produce pediocin and inhibit Gram-positive pathogens. In particular, pediocin produced from these strains have been found beneficial for inhibiting in the gastrointestinal tract the growth of Gram-positive pathogens of the enterococci type.

- 20 Alternatively, pediocin produced from the pediococci strains of the present invention will be obtained by purification from the culture supernatant of the pediocin-producing pediococci whilst the pediocin-producing pediococci will be obtained from the culture itself or via downstream processes which are known in the art such as centrifugation, filtration, washing and/or drying steps, etc.

- 25 Separate to the growth inhibitory properties of its bacteriocin, pediococci strains of the invention, preferably the pediocin-producing pediococci, and more preferably those isolated from human faeces have been found effective for inhibiting in the gastrointestinal tract the adhesion of Gram-negative strains.

- 30 Inhibition of pathogen adhesion in the gastro-intestinal tract is an important benefit provided by the pediococci of the invention, in particular the pediocin-producing pediococci and more preferably those isolated from human faeces. Hence, interaction between pathogenic bacteria and the host cells initiates infectious diseases. Attachment of these pathogens to intestinal cells is the first step of an infection. The pathogenic

bacteria may colonise, cause cell damage and cross the epithelial membrane. Pediococci strains of the invention and pathogens compete with each other on the binding sites of epithelial cells. Pediococci strains of the invention have been found able to prevent the binding of pathogens to the intestinal cells.

5

Preferred pediocin-producing pediococci are those of the type selected from *Pediococcus acidilactici*, *Pediococcus pentosaceus*, and mixtures thereof. Most preferred pediocin-producing pediococci are those of the *Pediococcus acidilactici* type.

10 Gram-positive species of the enterococci type that are preferentially inhibited by the pediococci according to the present invention are selected from vancomycin resistant enterococci, *Enterococcus faecalis*, *Enterococcus faecium*, and mixtures thereof.

15 Still, strains that are more preferentially inhibited by the pediocin-producing pediococci according to the present invention are vancomycin resistant enterococci, preferably vancomycin-resistant enterococci of *Enterococcus faecium* and/or vancomycin-resistant enterococci of *Enterococcus faecalis*.

Pediococcus acidilactici LMG P-21927

20 *Pediococcus acidilactici* LMG P-21927, that is *Pediococcus acidilactici* isolated from human faeces is a most preferred pediocin-producing *Pediococcus* strain for the purpose of the invention. Hence, the antimicrobial compound produced by the strain, i.e. pediocin has been found beneficial for inhibiting in the gastrointestinal tract strains selected from Gram-positive strains.

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Pediococcus acidilactici isolated from human faeces has been deposited at the Belgian Co-ordinated Collections of Microorganisms, Laboratorium voor Microbiology Gent (BCCMTM/LMG) on 24 April 2003 under No. LMG P-21927.

30 For the purpose of the present invention, when *Pediococcus acidilactici* LMG P-21927 is mentioned, this also encompasses its replicates and/or its derivatives.

By "replicate" is meant any biological material that represents a substantially unmodified copy of the materials, such as material produced by growth of microorganisms.

5 By "derivative" is meant material created from the biological material and which is substantially modified to have new properties, for example caused by heritable changes in the genetic material. These changes can either occur spontaneously or be the result of applied chemical and/or physical agents and/or by recombinant DNA techniques.

10 Separate to the growth inhibitory properties of its bacteriocin, *P. acidilactici* isolated from human faeces LMG P-21927 has been found beneficial for inhibiting in the gastrointestinal tract the adhesion of pathogenic strains, preferably Gram-negative strains.

15 Preferred Gram-positive species that are inhibited in growth are selected from vancomycin resistant enterococci, *Enterococcus faecium*, *Enterococcus faecalis*, *Listeria monocytogenes* and mixtures thereof. Still, the most preferred strains that are inhibited are vancomycin resistant enterococci.

20 Preferred Gram-negative species that are inhibited by the pediococci of the invention, in particular pediocin-producing pediococci and preferably *P. acidilactici* LMG P-21927, or pediocin produced from the pediococci supernatant, are selected from the genera *Klebsiella*, *Pseudomonas*, *Shigella* and mixtures thereof, and more preferably are selected from *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Shigella flexneri* and mixtures thereof. That such pediocin-producing pediococci do provide adhesion inhibition of Gram-negative pathogens while its bacteriocin provides growth inhibition of Gram-positive pathogens is surprising. Indeed, it is not common that both the bacteriocin and the respective bacteria will have an effect so that inhibition of both Gram-positive and Gram-negative strains are inhibited.

25

30 Further, the use of pediocin-producing pediococci, preferably *P. acidilactici* LMG P-21927, isolated from human faeces, as well as pediocin produced from these strains has been found effective for preventing associated disorders relating to the overgrowth

of pathogens in the GI tract, in particular the gut, namely diarrhea but also associated secondary disorders selected from water disturbances, mineral balance disturbances, malnutrition, dysfunctioning of tissues, dysfunctioning of organs, dysfunctioning of organism, and mixtures thereof. The disturbances and malnutrition can in turn cause 5 one or more of the following: nausea, vomiting, sickness but also endotoxemia, as well as sepsis ("blood-poisoning"), whereas the dysfunctioning mentioned above can in turn cause one or more of the following: death, infections, ulcers, bad wound healing both in the gut as well as topically.

10 Method of isolation and identification for the pediocin-producing pediococci

a)-Isolation

15 Faeces of healthy adult human volunteers are searched for probiotic strains. By "healthy", it is meant an adult human having no illness, no affliction, not suffering from the GI tract diseases, not having used antibiotics for at least 6 weeks, not having consumed probiotic products for at least a week, not intolerant to milk proteins, and having regular bowel habits. A diary concerning dietary habits was recorded.

20 Fresh human faeces is analysed in an anaerobic chamber. The faeces is diluted tenfold in 90 ml of storage medium (20 g/l buffered peptone water, 1.0 ml/l Tween 80, 0.5 g/l L-cysteine-HCl and 1 Resazurin tablet per liter, pH 6.3 (adjusted with 2M HCl)) and then homogenised by using an Ultra-Turrax. Serial dilutions are made in reduced physiologic pepton water and the 10^2 - 10^7 dilutions are plated on LAMVAB (Hartemink et al. 1997-LAMVAB-"A new selective medium for the isolation of lactobacilli from faeces, J. Microbiological methods 29,77-84). The low pH (5.0) in this medium inhibits the growth of Gram-negative bacteria and the Gram-positive bacteria vancomycin 25 resistant Lactobacilli and pediococci are resistant to vancomycin, so LAMVAB is selective for these strains. This medium consist of 104.4 g/l De Man Rogosa and Sharpe (MRS, Oxoid), 0.5 g/l L-cysteine-HCl, 0.05 g/l bromocresol green, 40 g/l agar, and 30 20 mg/l vancomycin. MRS, L-cysteine-HCl and bromocresol green are autoclaved separately from the agar for 15 minutes at 121°C and cooled down to 50°C. Vancomycin is sterilised by filtration using a 0.2- μ m filter. The three liquids are mixed

together and plates are poured. The plates are incubated at 37°C in anaerobic jars for three days. Gram-positive, catalase negative rod and coc shaped bacteria isolates are streaked for purity on MRS agar and incubated at 37°C.

- 5 Still, it has also been found that a medium composed of:
a)-a carbon and energy source that is not used by enterococci and the vast majority
of lactobacilli but which is used by pediococci, and
b)-an antibiotic which does not inhibit Pediococcus but does inhibit the other genera
such as an antibiotic derived from quinolones like Ciprofloxacin; and
10 c)-a small amount of pediocin to distinguish between a pediocin-producing
Pediococcus and a non pediocin-producing Pediococcus
provides a more selective medium for the determination of pediocin-producing
Pediococci.

- 15 Preferably, the selective medium for pediocin-producing pediococci is a modified
LAMVAB (Hartemink et al, 1997, as described hereinbefore) which contains 8g/l of
LAB-lemco powder (Oxoid) instead of meat extract given in LAMVAB, and a 2%
xylose as carbon source instead of glucose. Xylose is separately autoclaved and added
to the medium at 50°C. An antibiotic derived from quinolones such as Ciprofloxacin
20 (10mg/l) is added as an extra antibiotic and the supernatant of a steady state culture of
P. acidilactici LMG P-21927 (10%w/v) is added to inhibit the non-pediocin-producing
pediococci.

- 25 Accordingly, in a preferred embodiment, there is provided a medium for isolating
pediocin-producing Pediococci from substrate, which medium comprises xylose,
antibiotic derived from quinolones, and pediocin.

- b)-Selection for bacteriocin production
- 30 All isolated strains are then grown together with a positive control. *Lactobacillus curvatus* L530 in MRS for 24 hours at 37°C. Cells are removed by centrifugation (10 minutes, 4000 rpm, Sorval RT17), the pH of the supernatant is adjusted to 6.5 with NaOH and filtersterilised.

Indicator organisms were *Bacillus cereus* ATCC 11778, *Enterococcus faecium* ATCC 6569, *Klebsiella pneumoniae* LMD77-26, *Listeria monocytogenes* ATCC 7644, *Pseudomonas aeruginosa* DSM 1117 and *Staphylococcus aureus* ATCC 29213. These strains are grown overnight at 37°C in BHI-broth. Sterile BHI-agar of 50°C is
5 inoculated with 1% of indicator organisms and plates are poured. After solidifying, wells of approximately 5 mm are made and 50 µl of sterile supernatant of the isolated strains is added. After overnight incubation at 37°C, the diameter of the clear zones around the wells is measured.

10 c)-Species identification

The API 50CHL (BioMerieux SA, France) is used for tentative identification of the strains by their fermentation profiles. Cells are grown overnight on MRS agar plates. Cells are removed from the agar plate with a sterile swab and resuspended in the
15 suspension medium provided by the kit. API-strips are inoculated and analysed after 24 and 48 hours. Cells showing characteristics of a *Pediococcus* species are identified. Confirmation of cells being *P.acidilactici*, in particular *P.acidilactici* LMG P-21927 is confirmed with 16 sRNA sequencing.

20 d)-16sRNA

Sequencing of the 16sRNA gives a reliable identification of the strains. The extraction of the DNA of the strains is done according to the method described by Walter et al., 2000, "Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers", Applied and Environmental Microbiology, 66 (1), 297-303. The amplification and sequencing of the 16sRNA region is accomplished with primers mentioned in Table 1. The amplification program is 94°C for 5 min; 30 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 1 min 30 s; and finally 72°C for 4 min.
25
30

Table 1: Sequence primers

Sequence primer	Sequence (5' → 3')
8f	CAC GGA TCC AGA GTT TGA T(C/T)(A/C) TGG CTC AG
338r	GCT GCC TCC CGT AGG AG
338f	CTC CTA CGG GAG GCA GC
515f	TGC CAG CAG CCG CGG TAA TAC GAT
515r	ATC GTA TTA CCG CGG CTG CTG GCA
968f	AAC GCG AAG AAC CTT AC

Sequencing is carried out by the dydeoxy method of Sanger et al., 1977, "DNA sequencing with chain-terminating inhibitors", Proc. Natl. Acad. Sci. USA 74, 5463-5467, by using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems Inc., Nieuwekerk aan de IJssel, Netherlands) in combination with Applied Biosystems model 310 automated sequencing system. Analysis of nucleotide sequence data is carried out by using the Chromas/DNAasis program. The strain is identified with a BLAST search (NCBI), searching in the GenBank, EMBL, DDBJ and PDB databases.

Method of identification of the antimicrobial agent

- Protease resistance:
- To prove that the anti-microbial component is a protein, the activity of pediocin with addition of the enzyme protease K was tested with use of the well assay. *L. monocytogenes* ATCC 7644 was cultured 24 hours 37°C and re-cultured overnight in BHI at 37°C. BHI was autoclaved with 12 g/l agar and cooled down to 50°C. 1% inoculum of indicator organism *L.monocytogenes* ATCC 7644 was added to the agar. Plates were poured and allowed to solidify. Wells of approximately 5 mm in diameter were made, and 50-100 µl of serial dilutions of the filter sterile neutral supernatant of *P.acidilactici* LMG P-21927 was dispensed in duplo into the wells. At one corner of the well 3 µl of (10 mg/ml) of protease K was added. After overnight incubation at 37°C, the diameter of the clear zones around the wells was measured. If the anti-microbial component is a protein or peptide, the component will be broken down and the clear zone will be absent on the place where the protease K was added. With this

method it was demonstrated that the anti-microbial compound produced by *P.acidilactici* LMG P-21927 was a protein or peptide.

- Pediocin plasmid Isolation and sequence:

The plasmid with the pediocin-gene is isolated with use of the Birnboim method (Birnboim, HC and J.Doly, 1979, Nucleic Acid Res. 7:1513). The plasmid is multiplied with PCR, with use of the primers Pediocin 1 and Pediocin 2 (Table 2) and further purified with use of the GenElute PCR DNA purification kit (Sigma). A sequence PCR was done and the product is prepared for identification sequencing. After sequencing (use of the same primers, Table 2) the data is compared with data in the NCBI database. The plasmid of *P. acidilactici* LMG P-21927 has the gene that codes for pediocin PA-1/AcH.

Table 2: Primers pediocin

Primer	Sequence (5' → 3')
Pediocin 1	AAA ATA TCT AAC TAA TAC TTG
Pediocin 2	TAA AAA GAT ATT TGA CCA AAA

15

2)-Form of administration

Although the pediocin-producing pediococci according to the present invention can be used as such, it is preferred for use in the gastro-intestinal tract that the pediocin produced from *Pediococcus* is protected from its environment. Still for increased shelf-life storage, it may also be advantageous to protect the pediocin-producing pediococci strains, *P. acidilactici* LMG P-21927. The type of protection is not limited and can include coating, shell or encapsulation. The protection is designed for releasing its content, here the pediocin-producing pediococci, *P. acidilactici* LMG P-21927, or pediocin produced from these strains, directly to its target. This enables the delivery of pediocin-producing pediococci, *P. acidilactici* LMG P-21927, or pediocin produced from these strains in intact or almost intact form depending on the design of the protection to the gastro-intestinal tract, such as in the colon or if a slow release system is used in the whole GI tract.

20

25

A preferred mode of protection is by encapsulation. Method and material for encapsulation suitable for use herein are known in the art. Typical materials for encapsulation are selected from chitosan, maltodextrin, dextrans, lipids, polylactate, poly- or oligo saccharides, and mixtures thereof. Preferred encapsulating materials are selected from chitosan, maltodextrin, and mixtures thereof.

Accordingly, there is provided the use of pediocin-producing pediococci, *P. acidilactici* LMG P-21927, or pediocin produced from these strains in a protected form, preferably in encapsulated form.

10

A typical mode of administration to the patient as well as to healthy persons of the pediocin-producing pediococci, *P. acidilactici* LMG P-21927, or pediocin produced from these strains is enterally. A preferred enteral mode of administration is orally. Hence, when administered enterally, preferably orally, it is important that the strains or pediocin produced from these strains that are administered have a good survival rate so as to arrive in the small intestine with minimised destruction. By good survival, it is meant that at least 80% of the total ingested bacterial cells reaches the large intestine. The good survival is important as the strains have to survive the environment of the mouth, oesophagus, stomach, and finally small intestine but also when incorporated in products the strains have to survive the environment in the matrix of normal products that are meant for oral consumption. Of course, means of protection of the strains and/or bacteriocin as defined hereinbefore, such as encapsulation, can also be applied. It is still however important for the purpose of the invention that the strain fulfils the Survival Rate Test.

25

Probiotic

The strain of pediococci according to the present invention and preferably *Pediococcus acidilactici* isolated from faeces (LMG P-21927) as hereinbefore defined has been found effective when used as a probiotic. Accordingly, a probiotic component is provided, wherein the probiotic is a pediococci according to the present invention and preferably is *Pediococcus acidilactici* isolated from faeces (LMG P-21927). Advantageously, the invention probiotic has a good survival rate in the stomach as well as in the intestine which is comparable to other known probiotics, such as *L. rhamnosus*.

ATCC 7469, *L. rhamnosus* ATCC 53103, *L. plantarum* DSM 9843, *B. animalis* from Christian Hansen or better than known probiotics, such as *L. acidophilus* La5 Christian Hansen, *L. reuteri* LMG 9213.

5 Nevertheless, when better performance and/or shelf life of the invention probiotic is desired, protection of the probiotic as defined herein before, preferably by encapsulation, can be made.

Health promoting action composition

10 A health promoting action composition is also herein provided. The invention composition thus comprises a probiotic component, the probiotic component being a pediococcus according to the invention, and more preferably is *P. acidilactici* isolated from human faeces LMG P-21927, all of which being defined hereinbefore, and preferably additionally comprising one or more of the selected components: additional
15 probiotic, prebiotic, immunoglobulines, and mixtures thereof.

Alternatively, the probiotic component can be replaced by the pediocin produced from the probiotic strain or the pediocin can be used in combination with the probiotic in the health promoting action composition of the invention.

20 When used, the pediocin produced from the probiotic strain and present in the supernatant of the strain will be administered at a dosage of from 50 to 1000 millilitres pure supernatant per day, preferably from 200 to 500 ml. Additionally, the supernatant can be concentrated or the pediocin partly purified, subsequently leading to a lower
25 dosage, dependent on the extent of concentration.

When present, the amount of probiotic component within the invention composition will be present in an amount of from 10^6 to 5×10^{11} cfu/day, preferably from 10^8 to 10^{10} cfu/day.

30

- Additional probiotic

The invention composition can comprise an additional probiotic. By use of an additional probiotic, the spectrum of activity can be further broadened or the activity of

the *P. acidilactici* LMG P-21927 can even be increased. Preferred additional probiotics are selected from *Lactobacillus* strains, *Bifidobacterium* strains, *Propionibacterium* strains and mixtures thereof. More preferred additional probiotics are selected from *L. rhamnosus*, *L. plantarum*, *B. animalis*, *B. lactis*, *L. fermentum*, *B. adolescentis*, *L. 5 acidophilus*, *L. reuteri*, *B. longum*, *B. infantis*, *B. bifidum*, *B. breve*, *L. casei*, *L. johnsonii*, *L. gasseri*, *L. crispatus*, *L. helveticus*, *L. salivarius*, *L. lactis*, *L. brevis*, *L. paracasei*, *L. sakei* and mixtures thereof.

When present, the amount of additional probiotic component within the invention 10 composition will be present in an amount of from 10^6 to 5×10^{11} cfu/day, preferably from 10^8 to 10^{10} cfu/day.

- Prebiotics

Prebiotics are substances that form a substrate for the probiotic compound. As a 15 result, the likelihood that the microorganisms forming the probiotic component reach the intestines alive increases. Further, the combination of prebiotic with the probiotic enable increase of the beneficial action of the growth of beneficial bacteria such as probiotic components. Accordingly, prebiotics can advantageously be used in the invention composition. Suitable prebiotics for use herein are selected from 20 transgalactooligosaccharide, hydrolysed Locust bean gum, inulin, hydrolysed inulin, fructooligosaccharides, xylooligosaccharides, xylopolyolsaccharides and mixture thereof. Preferred prebiotics are selected from inulin, hydrolysed inulin, fructooligosaccharides and mixtures thereof.

25 When present, the amount to be administered of prebiotic component will be of from 0.05 to 20 g/day, preferably from 0.2 to 10 g/day, most preferably from 0.5 to 5 g/day.

- Immunoglobulines

Immunoglobulines are also suitable ingredients for use herein. Use of these in the 30 invention will provide an additional or even synergistic effect on the reduction or inhibition of VRE. Preferably, the use of immunoglobulin Y from bird eggs is used. More preferably, the poultry has been hyperimmunised with pathogenic bacteria. Most preferably immunoglobulin Y derived from eggs of birds hyperimmunised against

enterococci, *Pseudomonas*, *Klebsiella*, *Shigella* and mixtures thereof are used. Immunoglobulins can be added as in the egg fraction and/or be (partially) purified. Immunoglobulin Y interacts with the pathogenic bacteria in such a way that they are less able to colonise the GI tract of the host.

5

When proteins are used that originate from eggs of hyperimmunised birds, the daily dose of IgY that can be administered is then preferably from 0.2 to 1200 mg, more preferably within the range of 0.5 to 800 mg, most preferably from 10 to 600mg. Stated otherwise, the dose of the ingredient is preferably of from 0.003 to 20 mg per dose per kg body weight, more preferably from 0.08 to 13 mg per dose per kg body weight, most preferably from 0.15 to 10 mg per dose per kg body weight.

10

Form of the composition

15

The invention composition can be in any form which is suitable for its end use. This includes liquid, paste or solid form such as powder form.

The invention composition can be packaged in various forms including sachet, capsule, bar, suppository.

20

Administration of the invention composition or compound

The present invention is enterally administered. However, preferred modes of administration are by tube feeding or orally, more preferably oral administration.

25

The following are non-limiting examples according to the present invention

Example 1-Survival in static stomach and small intestine model

The survival in the stomach and small intestine of *P. acidilactici* isolated from human faeces LMG P-21927 as well as known probiotic strains was evaluated. The survival of *P. acidilactici* isolated from human faeces LMG P-21927 in the stomach and small intestine is important when the strain is used as a probiotic in humans.

30

Besides *P. acidilactici* LMG P-21927, known *Pediococcus* strains were tested: *P. acidilactici* ATCC 8081, *P. acidilactici* from a starter culture for meat fermentation from Christian Hansen, and *P. acidilactici* DSM 20284.

5 The bacteria were grown in MRS for 24 hours and subsequently re-inoculated for 18 hours in MRS. 1 ml of the grown culture was added to 9 ml of the stomach medium, consisting of 8.3 g/l bacteriological peptone, 3.1 g/l NaCl, 0.11 g/l CaCl₂, 1.1 g/l KCl, 0.6 g/l KH₂PO₄, 22.2 mg/l pepsin and 22.2 mg/l lipase, pH 3.0. The bacteria were incubated for 3 hours at 37°C in the stomach medium. Afterwards 1 ml of the incubated
 10 stomach medium with the bacterium was mixed with 9 ml of small intestine medium and incubated for another 3 hours at 37°C. The small intestine medium consists of 5.7 g/l bacteriological peptone, 1.25 g/l NaCl, 0.055 g/l CaCl₂, 0.15 g/l KCl, 0.68 g/l KH₂PO₄, 1.0 g/l NaHCO₃, 0.3 g/l Na₂HPO₄, 0.7 g/l glucose, 20.3 g/l pancreatin and 5.5 g/l bile, pH 6.5. Samples were taken at t=0, 3, and 6 hours and plated on MRS agar to
 15 determine the colony forming units.

20 *P. acidilactici* isolated from human faeces LMG P-21927 presented similar and good survival in the stomach medium as the *P. acidilactici* isolated from plant. However, it was found that *P. acidilactici* isolated from human faeces LMG P-21927 presented a better survival in the small intestine medium than the *P. acidilactici*
 25 isolated from the other sources (Table 3).

Table 3: Survival of pediococci in small intestine medium (% of the colony forming units). The pediococci were first incubated in stomach medium for 3 h, after which the survival was set to 100 %.

Strain	Origin	Survival small intestine
<i>P.acidilactici</i> LMG P-21927	Human faeces	107
<i>P.acidilactici</i> ATCC 8081	Fermented milk	73
<i>P.acidilactici</i> DSM 20284	Wheat	58

Example 2

The *P.acidilactici* strains LMG P-21927, ATCC 8081 and DSM 20284 were grown in MRS for 24 hours and subsequently re-inoculated for 18 hours in MRS. The grown culture was 10 times diluted in PPS (Peptone physiological salt solution) (8,5 g/l sodium chloride, 1 g/l bacteriological peptone). 1 ml of this dilution was added to 9 ml of small intestine medium, with 5.0 or 1.1g/l bile and incubated for 3 hours at 37°C. Samples were taken at t= 0, and 3 hours and plates out on MRS agar to determine the colony forming units. Results showed that *P.acidilactici* LMG P-21927 survived the small intestine with different bile salt concentrations better than the other pediococci (Table 4). This indicates that a pediococcus isolated from human faeces has better probiotic properties than a pediococcus selected from food and plants, since it has an improved ability to survive the conditions of the small intestine.

Table 4: Survival (in % of the colony forming units) of pediococci in small intestine medium with 0.1%, and 0.5 % Bile without prior incubation in stomach medium.

Strain	Origin	0.1%	0.5%
<i>P.acidilactici</i> LMG P-21927	Human faeces	163	155
<i>P.acidilactici</i> ATCC 8081	Fermented milk	103	135
<i>P.acidilactici</i> (Christian Hansen)	Starter culture for meat fermentation	92	115
<i>P.acidilactici</i> DSM 20284	Wheat	97	73

Example 3-Growth on prebiotics

P. acidilactici LMG P-21927 was grown in MRS for 24 hours and subsequently re-inoculated for 18 hours in MRS. Cultures were harvested by centrifugation (10 minutes 4000 rpm, Sorval RT 17) and the pellet was washed with and resuspended in PPS. This step was repeated. M17 medium (Oxoid) was prepared and different fibres were used as carbon source. The different M17 media were inoculated with 1% with the washed

bacteria and incubated during 24 hours at 37°C. During incubation the optical density was measured.

Results showed that *P. acidilactici* LMG P-21927 could grow on: trans-galactooligosaccharides (0.5% w/v), hydrolysed Locust bean gum (0.5% w/v) and inulin, fructo-oligosaccharides and hydrolysed inulin (0.5% w/v).

Example 4-Bacteriocin activity

Growth and production of pediocin

The pediocin used for these studies was prepared as follows:

10 *P. acidilactici* LMG P-21927 isolated from human faeces was grown for 24 hours 37°C in MRS. 1% reinoculum in MRS was incubated for 24 hours at 37°C. The cells were removed by centrifugation (10 minutes, 4000 rpm, Sorval RT17). With NaOH the supernatant was set to pH 6.5 and after that filter-sterilised and frozen (-20°C) until use. Supernatant of *P. acidilactici* DSM 20284 was used as a control, because this strain did 15 not produce pediocin.

Detection of the antimicrobial activity

The bacteria tested for sensitivity for *P. acidilactici* LMG P-21927 were Gram-positive pathogens *Listeria monocytogenes* ATCC 7644, *Enterococcus faecium* (ATCC 20 6569-non VRE), *Enterococcus faecium* (LMG 21895, LMG 21896, LMG 21897, ATCC 700221, LMG 21898, LMG 21899, LMG 21900 which are all VRE clinical isolates of *Enterococcus faecium*), *Enterococcus faecium* ATCC 700221 (VRE), *Enterococcus faecalis* (ATCC 4200, LW603, ATCC 376, ATCC 14428, ATCC 29212, all non VRE) and commensal bacteria and probiotic strains *Lactobacillus rhamnosus* 25 ATCC 7469, *Lactobacillus acidophilus* LW 74-2, *Lactobacillus casei* DSM20011, *Lactobacillus plantarum* DSM20174, *Lactococcus lactis* LW53, *Bifidobacterium longum* BB536, *Bifidobacterium breve* ATCC 15700, *Bifidobacterium animalis* Bb12 from Christian Hansen, *Bifidobacterium adolescentis* ATCC 15705 and *Bifidobacterium bifidum* DSM20456.

30

The pathogens are grown in Brain Heart Infusion (BHI, Oxoid) and the lactobacilli, lactococci and bifidobacteria in MRS. The bifidobacteria were grown in an anaerobic chamber in MRS with 0.5 g/l cysteine-HCl and 1 resazurin tablet per liter medium.

The antimicrobial activity of *P.acidilactici* LMG P-21927 was detected using a well diffusion assay and by measuring the optical density (OD) during growth. The bifidobacteriae were only tested with the well diffusion assay, because of difficulties in measuring the OD under anaerobic conditions.

5

In the well diffusion assay the bacteria were grown in their standard medium for 24 hours at 37°C and re-cultured overnight at 37°C. Suitable media were autoclaved with 12 g/l agar and cooled down to 50°C. 1% inoculum of the to be tested bacteria was added to the agar. Plates were poured and allowed to solidify. Wells of approximately 5 mm in diameter were made, and 50-100 µl of serial dilutions of the filter sterile neutral supernatant of *P. acidilactici* LMG P-21927 was dispensed in duplo into the wells. After overnight incubation at 37°C, the diameter of the clear zones around the wells was measured.

10

In the other method the OD at 600 nm was measured in time for bacteria incubated with supernatant of *P.acidilactici* LMG P-21927. Bacteria were grown in their normal medium for 24 hours at 37°C and re-inoculated 1% in the same medium for 18 hours at 37°C. 125 µl of supernatant of *P. acidilactici* LMG P-21927 was mixed in a 100-wells-plate with an equal amount of double concentrated media of the bacteria to be tested.

15

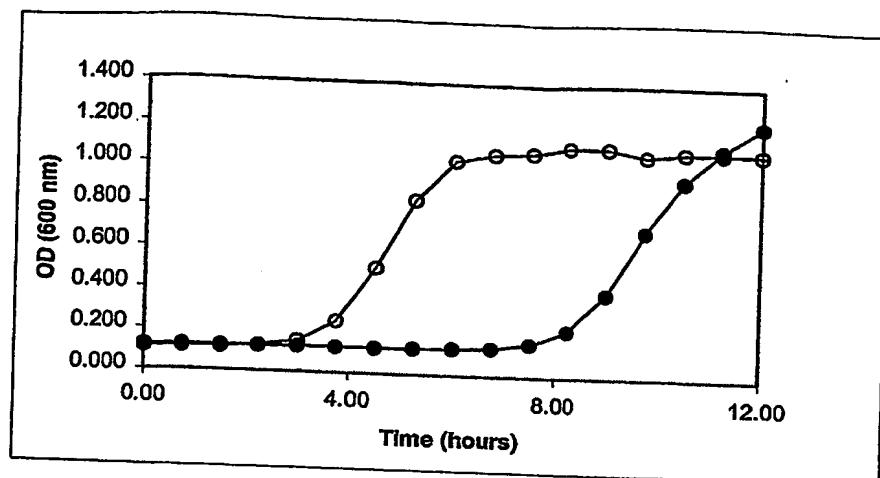
The bacteria were added so that the end concentration in the plates was 10^5 cfu/ml. OD_{600nm} was measured during 24 hours at 37°C with the use of a Bioscreen apparatus. Bacterial growth with supernatant of *P. acidilactici* DSM 20284 was used as a control. Experiments were done in duplo.

20

Results

Listeria monocytogenes ATCC 7644 and *E.faecium* strains both VRE and non-VRE and *E.faecalis* strains were sensitive for supernatant of *P.acidilactici* LMG P-21927. *L.monocytogenes* ATCC 7644 was inhibited by *P.acidilactici* LMG P-21927 by lowering the growth rate and *E.faecium* had a much longer lag-phase than without the supernatant (Figure 1). Commensal and probiotic strains such as lactobacilli and bifidobacteria were not sensitive for *P.acidilactici* LMG P-21927 and its pediocin.

30



5 Figure 1: Effect of supernatant of LMG P-21927 containing pediocin (closed symbols) and a control supernatant from a *P. acidilactici* which does not produce pediocin (open symbols) on growth of a vancomycin resistant strain of *E. faecium* as determined by optical density.

10 To measure if the effect of *P. acidilactici* LMG P-21927 on the bacteria was bactericidal or bacteriostatic time-kill curve studies were done. Supernatant of *P. acidilactici* LMG P-21927 was mixed 1:1 with 2 times concentrated BHI. 10^5 cfu/ml of *L. monocytogenes* ATCC 7644 or VRE *E. faecium* (LMG 21895, LMG 21896, LMG 21897), *E. faecium* ATCC 700221 VRE was added to medium and supernatant. Samples were taken during appropriate intervals for a period of 24 hours period to determine the viable cell count.

15 The effect of *P. acidilactici* on bacteria is bactericidal. The amount of *E. faecium* is decreased 1Log unit after adding supernatant. (Figure 2).

5

10

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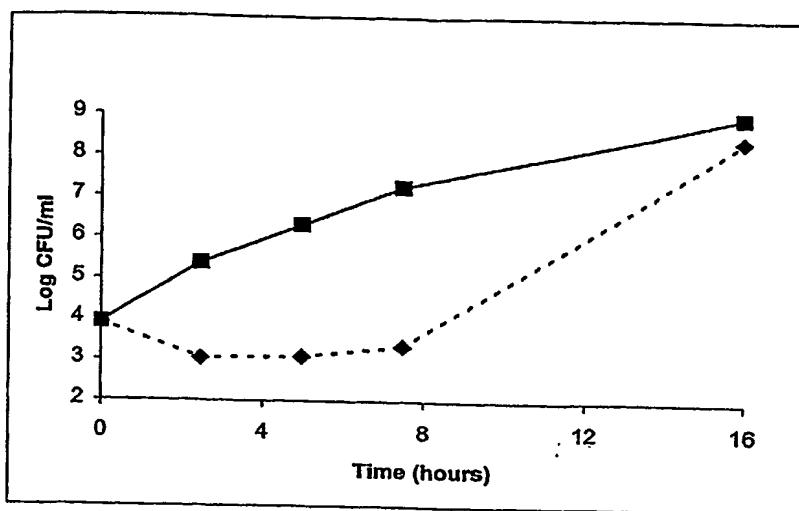


Figure 2: Effect of supernatant from LMG P-21927 containing pediocin (dotted line) compared to the effect of the control supernatant of a non-pediocin-producing *Pediococcus acidilactici* (straight-line) on growth of a vancomycin resistant strain of *E. faecium* strain as determined by cfu/ml.

Example 5- Adhesion of *P.acidilactici* to Caco-2 cells compared with known probiotic strains.

One of the properties of probiotics is that they can adhere to intestinal cells and compete with pathogens for the binding sites of the epithelial cells.

The adherence of well known, good adhering probiotic strains *Lactobacillus rhamnosus* ATTC 53103 (L GG), and *Bifidobacterium animalis* Bb-12 (Christian Hansen), and of *P.acidilactici* LMG P-21927 was tested.

Overnight cultures of the strains were harvested by centrifugation (10 minutes, 4000 rpm, Sorval RT17) and resuspended in PBS. The amount of cells was counted under a microscope with use of a Bürker Türk counting chamber. Bacteria were centrifuged again and the pellet was resuspended in Caco-2 1% FCS-medium Pen/Strep free. The Caco-2 cells were 2 weeks post-confluence and grown in 24 wells-plate ($2.5 \cdot 10^5$ Caco-2 cells per well). Per well $2.5 \cdot 10^8$ CFU of the bacteria were added and incubated for 1 hour at 37°C in an incubator with 5% CO₂. After incubation the media was removed from the Caco-2 cells and the cells were washed 3 times with PBS (37°C). Cells were lysed with sterile Mili Q water, serial dilutions of the lysed cells were made and plated on MRS agar.

Results showed that *P.acidilactici* LMG P-21927 adhered better than other probiotic strains (Table 5).

5 Table 5: Adhesion of protiotics to Caco-2 cells.

	Adhesion (% of total bacterial cells added)
<i>P.acidilactici</i> LMG P-21927	0,9
<i>L.rhamnosus</i> ATTC 53103	0,14
<i>B.animalis</i> BB12 (Christian Hansen)	0,66

10 Example 6-Inhibition of adhesion and/or invasion of pathogenic bacteria by *P.acidilactici* LMG P-21927

Interaction between pathogenic bacteria and the host cells initiates infectious diseases. Attachment of these pathogens to intestinal cells is the first step of an infection. The pathogenic bacteria may colonise, cause cell damage and when invasive cross the epithelial membrane. Probiotic strains and pathogens compete with each other 15 on the binding sites of epithelial cells. Probiotic strains can prevent the pathogens in binding to the intestinal cells.

Using cultured Caco-2 cells as a human intestinal cell model, the adhesion of pathogens and probiotic strains to cells (Coconnier, et el., 1993) was tested. Pathogens 20 tested were *Klebsiella pneumoniae* LMG 21902, *Pseudomonas aeruginosa* LMG 21901 and *Shigella flexneri* LMG 21935, all deposited at the Belgian Co-ordinated Collections of Microorganisms, Laboratorium voor Microbiology Gent (BCCMTM/LMG).

25 The probiotics used were *P.acidilactici* LMG P-21927 and the commercial used strain *Lactobacillus rhamnosus* ATTC 53103 and *Bifidobacterium animalis* Bb-12.

Overnight cultures of pathogens and probiotics were harvested by centrifugation (10 minutes, 4000 rpm, Sorval RT17) and resuspended in PBS. The amount of cells was counted under a microscope with use of Bürker Türk counting chamber. Bacteria were centrifuged again and the pellet was resuspended in Caco-2 1% FCS-medium Pen/Strep free. The Caco-2 cells were 2 weeks post-confluence and grown in 24 wells-plate (2,5 10⁵ Caco-2 cells per well). Per well 5 10⁷ CFU of the pathogens and 2,5 10⁸ CFU of the probiotic bacteria were added and incubated for 1 hour at 37°C in an incubator with 5% CO₂. After incubation the media was sucked off from the Caco-2 cells and the cells were washed 3 times with PBS (37°C). Cells were lysed with sterile Mili Q water, serial dilutions of the lysed cells were made and plated on Nutrient agar (for pathogens) and on MRS agar (for probiotic strains).

P.acidilactici LMG P-21927 was found to reduce the adherence of the pathogens to intestinal cells. *P.acidilactici* LMG P-21927 was found as good in prevention of adhesion as well known probiotics (Table 6).

Table 6: Inhibition of adhesion of pathogens to Caco-2 cells by probiotics (%).

	<i>K.pneumonia</i> LMG 21902*	<i>P.aeruginosa</i> LMG 21901*	<i>S.flexneri</i> LMG 21935*
<i>P.acidilactici</i> LMG P-21927	40	35	25
<i>L.rhamnosus</i> LGG ATTC 53103	44	38	nd
<i>B.animalis</i> BB12 (Christian Hansen)	nd	42	nd

* The prevention (%) of the adhesion of the Caco-2 cells by probiotic bacteria:
 Difference in adherence of the pathogens with and without probiotic strains. Nd = not determined.

Example 7

A product in the form of a sachet containing 1 g of maltodextrin and 5×10^9 cfu *Pediococcus acidilactici* LMG P-21927.

10 The sachet product is to be taken twice a day.

The contents of the sachet are intended for addition to a beverage or dessert and mixed upon consumption. The product, to which it is added is cold or lukewarm, but does not have a temperature exceeding 45 °C.

15 Example 8

Milk powder or infant milk formula (powder) containing 1×10^7 cfu *Pediococcus acidilactici* LMG P-21927 per gram. The powder further contains a probiotic belonging to the genus *Bifidobacterium* in a concentration of 1×10^7 cfu/g.

20 Example 9

Capsule, with an acid resistant coating, containing 2×10^9 cfu *Pediococcus acidilactici* LMG P-21927, 0.4 g of hydrolysed inulin and 0.1 g of protein derived from eggs of chickens, which were hyperimmunised against a cocktail of vancomycin resistant *Enterococci* strains.

Example 10

Milk powder, obtained after fermentation of skim milk, supplemented with 0.5 wt % glucose, and *Pediococcus acidilactici* LMG P-21927 at a pH kept constant at 6.0. After 48 h of fermentation at 37 °C, the fermented milk is dried by processes known in the art.

Example 11

Probiotic bar of 23 gram containing 4.0 g oat flakes, 3.0 g wheat flakes, 3.0 g puffed rice, 1.0 g crushed hazelnuts, 0.25g raisins, 1.5 g maltodextrin, and 2×10^9 cfu *Pediococcus acidilactici* LMG P-21927.

Example 12

Isotonic drink with a sealed cap that contains 1.0 gram maltodextrin and 2×10^9 cfu *Pediococcus acidilactici* LMG P-21927.

CLAIMS

1. Use of pediococci for the manufacture of a composition for inhibiting pathogenic strains in the gastrointestinal tract, wherein the pediococci are characterised by a survival rate as per the Survival rate Test defined herein of at least 80 %, preferably of at least 90 %, most preferably of at least 96 %.
5
2. Use according to Claim 1, wherein the pediococci are pediocin-producing pediococci.
10
3. Use according to either one of Claims 1 or 2, wherein the pediococci are isolated from human faeces.
- 15 4. Use according to any one of Claim 1-3, wherein the pediococcus is *P. acidilactici* LMG P-21927 and/or replicates and/or its derivatives thereof.
- 20 5. Use of pediococci as defined in any one of Claims 1-4 for the manufacture of a composition for inhibiting in the gastrointestinal tract the adhesion of pathogen strains of the Gram-negative type, preferably Gram-negative strains of the genera selected from *Klebsiella*, *Pseudomonas*, *Shigella* and mixtures thereof; more preferably selected from *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Shigella flexneri* and mixtures thereof.
25
6. Use of pediococci and/or bacteriocin produced from pediococci as defined in any one of Claims 1-4 for the manufacture of a composition for inhibiting in the gastrointestinal tract the growth of Gram-positive strains of the genus Enterococci, preferably Gram-positive strains selected from vancomycin-resistant enterococci, *Enterococcus faecium* and mixtures thereof, more preferably vancomycin-resistant enterococci.
30
7. Use of pediococci and/or bacteriocin produced from pediococci as defined in either one of Claims 3 or 4 for the manufacture of a composition for inhibiting in the

gastrointestinal tract the growth of Gram-positive strains, preferably Gram-positive strains selected from vancomycin resistant enterococci, *Enterococcus faecium*, *Enterococcus faecalis*, *Listeria monocytogenes* and mixtures thereof, more preferably vancomycin resistant enterococci.

5

8. Use according to any of the preceding claims, wherein the pediococci, or pediocin produced from these strains is in encapsulated form.

10 9. Use according to any of the preceding claims, wherein the pediococci, or pediocin produced from these strains is enterally administered to the patient, preferably orally.

15 10. Use of pediococci as defined in any one of claims 1-4 or pediocin produced from these strains for the manufacture of a composition for preventing and/or treating diarrhoea and/or secondary disorders associated with overgrowth of pathogens or infection by pathogens in the gastrointestinal tract, the secondary disorders being preferably selected from water disturbances, mineral balance disturbances, malnutrition, dysfunctioning of tissues, dysfunctioning of organs, dysfunctioning of organism, and mixtures thereof.

20 11. A probiotic component, wherein said probiotic component is a pediococci as defined in any one of claims 1-4.

25 12. A health promoting action composition comprising a probiotic component as defined in Claim 11 and preferably further comprising a component selected from pediocin as defined in claim 6 or 7, additional probiotics, prebiotics, immunoglobulines, and mixtures thereof.

30 13. A health promoting action composition comprising pediocin as defined in any one of claims 1-7 and preferably further comprising a component selected from a probiotic component as defined in Claim 11, additional probiotics, prebiotics, immunoglobulines, and mixtures thereof.

14. A component according to Claim 11 or composition according to either one of Claim 12 or 13, wherein the probiotic component and/or pediocin produced from the probiotic component is in encapsulated form.
- 5 15. A component according to Claim 11 or composition according to any one of Claims 12-14, wherein the component or composition is enterally administered to the patient, preferably orally.
- 10 16. A medium for isolating pediocin-producing Pediococci from a substrate, which medium comprises xylose, antibiotic derived from quinolones, and pediocin.
17. A method for isolating pediocin-producing Pediococci from a substrate, wherein the pediocin-producing Pediococci is isolated by a medium as defined in Claim 16.
- 15 18. A medium according to Claim 16 or method according to claim 17, wherein the substrate is human faeces.

ABSTRACT

There are provided pediocin-producing pediococci that have a good survival property in the small intestine as well as pediocin produced thereof, in particular
5 *Pediococcus acidilactici* isolated from human faeces for use in the gastrointestinal tract of humans to provide a health promoting action, in particular against infection by multi-resistant pathogens. Also provided herein are compositions incorporating such pediocin-producing pediococci.

13. 06. 2003

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